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An Open Cephalic Neural Tube Reproducibly Induced by Cytochalasin D in Rat Embryos *in vitro*

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ABSTRACT—Rat embryos at the head-fold stage (Slc : SD strain; 9.5 days of gestation) were cultured in rat serum in the presence of a relatively low concentration of cytochalasin D (2×10^{-8} M). Embryos developed into C-shaped structures (with the open part of the C directed ventrally) with an open cephalic neural tube. Elevation and apposition of the neural folds and eversion of the neural plates at the procencephalon, mesencephalon and some parts of rhombencephalon were observed during the course of development of the treated embryos. In control embryos, staining with rhodamine-conjugated phalloidin was observed at the basal region of the lateral margin of the fusing neural folds and at the luminal surface of the roof plates of the procencephalic neural tube. The latter diminished as embryos developed. However, the staining was confined to the edges of the apposed neural folds in the treated embryos and became intense at the luminal surface of the everted neural plates. By contrast, some parts of the rhombencephalon of the treated embryos showed fusion of the neural folds. The staining was observed in roof plates of the rhombencephalon in these embryos as like as control embryos. The area of the staining at the luminal surface of the roof plates spread as the fourth ventricle expanded. These results suggest that microfilaments do not play an essential role in the elevation of the neural plates but do play an important role in fusion of the neural folds and the moulding of the cephalic neural tube. Delicate changes in the distribution of microfilaments may result in changes in cell shape that cause the fusion of the neural folds and the moulding of the cephalic neural tube.

INTRODUCTION

Microfilaments are intimately associated with neurulation in amphibian, avian and mammalian embryos [5, 6, 9, 16, 19, 20, 23]. The growth of microfilaments in inhibited and preformed microfilaments are fragmented by treatment with cytochalasins [24]. Among the cytochalasins, cytochalasin D has a particularly high affinity for contractility-related binding sites but it has no effect on hexose transport [3, 11, 26–27]. Therefore, not unexpectedly, treatment of mammalian embryos with cytochalasin D induces abnormal neurulation.

Cytochalasins frequently induce exencephaly in fetuses when administrated to pregnant dams [1, 22, 30–31]. The concentrations of cytochalasin D, injected intraperitoneally, that induce exencephaly at high frequency range from 0.7 to 1.5 mg/kg. Some embryos from mothers treated with cytochalasins have an open neural tube, in which the everted cranial neuroepithelium can be seen [1, 22]. However, many other embryos from treated dams have other major malformations, such as spina bifida, tail defects, eye defects, and ear defects. Therefore, it is difficult to investigate the correlation between microfilaments and abnormal neurulation in such a system *in vivo*.

In experiments *in vitro*, Morriss-Kay and Tuckett [13] found that, in cytochalasin D-treated rat embryos, the neural folds lost their apical constriction, the elevated neural folds were flattened, and the neural folds collapsed. These embryos had been exposed to a relatively high concentration of cytochalasin D (3×10^{-7} M). Similar doses have been used in other systems [3, 11, 25, 29, 32–33]. However, when exposed to cytochalasin D at this rather high concentration (3×10^{-7} M), embryos of C57BL/6 mice die within 24 hr [17]. It is not clear that a culture system in which embryos have collapsed neural folds is useful as a model system for investigations of neurulation. Therefore, a system is needed in which embryos all show the same type of neural malformation, such as an open neural tube. We have cultured rat embryos in a medium that contains a relatively low concentration of cytochalasin D (2×10^{-8} M) and, under our conditions all the embryos develop an open cephalic neural tube.

In this report, we describe the morphological features of the open cephalic neural tube in cytochalasin-treated rat embryos, as well as the pattern of distribution of microfilaments during failure of closure of the cephalic neural tube. Possible causes of the anomaly are discussed.

MATERIALS AND METHODS

Embryo culture

Female Sprague-Dawley (SD) rats (Slc : SD strain; Japan SLC., Shizuoka, Japan) were caged overnight with males in breeding rooms with a 12-hr light cycle, constant temperature (24 ± 2°C) and constant humidity (55 ± 10%). Noon of the day on which a vaginal plug was observed was designated as day 0.5 of gestation. Embryos were cultured according to the methods of New et al. [15]. Embryos at the head-fold stage (9.5 days of gestation, 1.6–1.8 mm in egg cylinder length) were disssected from the uterus in Hanks' solution. After removal of the decidua and rupture of Reichert's membrane, four embryos at a time were put into a culture bottle that contained 4 ml

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of rat serum. Serum was obtained by immediate centrifugation of blood after its withdrawal from SD rats; it was inactivated by heating at 56°C for 30 min. Streptomycin sulfate and benzyl penicillin, sodium salt (Meiji Seika Kaisha, Tokyo, Japan) were added to final concentrations of 1.4×10^{-4} M and 1.8×10^{-4} M, respectively. The culture bottles were gassed initially with 5% O_2, 5% CO_2, and 90% N_2, which was replaced with 20% O_2, 5% CO_2, and 75% N_2 after 22 hr of culture. Finally, the gas was replaced with 40% O_2, 5% CO_2, and 55% N_2 after 24 hr. The bottles were incubated at 37°C on a rotator (40 rev/min) throughout the culture period. Cytochalasin D (Sigma, St Louis, MO, USA), dissolved in dimethylsulfoxide (DMSO; Katayama Chemical Co., Osaka, Japan), was added to the culture medium to a final concentration of 2×10^{-8} M. The concentration of DMSO used in this study (6.4×10^{-8} M, final concentration) did not affect the development of rat embryos, as reported previously by Kitchin and Ebron [7]. Embryos were observed at intervals under a binocular microscope and fixed overnight in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) at 4°C.

Scanning electron microscopy

Fixed embryos were dehydrated in a graded alcohol series, critical-point dried, mounted on brass stubs with electron-conductive paint (Dotite; Fujikura Kasei, Co., Ltd., Tokyo, Japan), coated with gold in a sputter coater, and observed with a scanning electron microscope.

Light microscopy

Transverse and sagittal 10-μm sections of embryos were prepared by the method of Matsuda [10]. Sections were put on slide glasses, deparaffinized and stained with Mayer's hemalaum. They were enclosed in Entellan (new; E. Merck, AG., Darmstadt, Germany) and examined under a light microscope.

Histochemical procedures

Transverse and sagittal 10-μm sections of embryos were put on slide glasses and deparaffinized. For histochemistry to phalloidin, deparaffinized sections were washed with 20 mM Tris buffer that contained 0.15 M NaCl (pH 7.5), incubated for 30 min at RT with rhodamine-conjugated phalloidin (Wako chemical Ind., Ltd., Osaka, Japan) that had been diluted 1:20 in the buffer. Sections were washed three times with the buffer, enclosed in glycerin jerry (7% gelatin in 50% glycerin containing 0.02% sodium azide) and examined under a fluorescence microscopy (Fluophoto; Nikon, Tokyo, Japan).

RESULTS

Gross and microscopic observations

When rat embryos were cultured for 48 hr in rat serum that contained 2×10^{-8} M cytochalasin D, embryos became C-shaped, with the open part of the C directed ventrally. Their development was almost the same as that of control embryos except in the head region (Table 1). Their cephalic neural plates remained open at the prosencephalon, mesencephalon and rhombencephalon, as shown in Figure 1B. Cephalic neural plates fused in 33-hr-cultured control embryos. However, only elevation and apposition of the neural folds but not fusion were observed in the course of the development of the cytochalasin D-treated embryos (Fig. 2A). The cephalic neural plate was everted in 48-hr-cultured treated embryos (Fig. 2B) and the diencephalon protruded between and over the telencephalic vesicles so that the parts that normally formed the roof of the ventricle were continuous with the skin of the temporal area (Fig. 3A). Transverse sections of the treated embryos showed that the neural plate protruded into the ventricle in the telencephalon (Fig. 3C). A wide space between the neuroepithelium and surrounding mesenchymal cells can be seen in Figure 3C. Anomalies in the neural plate of treated embryos were rather limited in the rhombencephalon (Fig. 3E), as compared with the prosencephalon and the mesencephalon, and part of the rhombencephalon, adjacent to the mesencephalon, showed failure of closure of the neural tube. The rhombencephalic neuroepithelium continued to the neural groove, with both sides closely apposed, in the treated embryos (Fig. 3E) but not in the control embryos (Fig. 3D). The ventricle was more spacious in control embryos than in treated embryos. Trigeminal and facio-acoustic neural crest complexes were observed in the treated embryos as they were in the control embryos (data not shown).

Histochemical observations

In the telencephalic region, neural plates elevated, apposed and everted in cytochalasin D-treated embryos, although control embryos showed fusion of the neural folds and formation of the ventricle. In the 24-hr-cultured control embryos, staining with rhodamine-conjugated phalloidin was observed at the basal region of the lateral margin of the fusing neural folds (Fig. 4A). By contrast, the staining was observed at the surface of the end region of the neural plates

Table 1. Effects of cytochalasin D on rat embryos cultured *in vitro* from day 9.5 of gestation for 48 hr

	No. of embryos	No. of embryos with open cephalic neural tube	No. of somites	Crown-rump length (mm)	Head length (mm)
Control	15	0	26.5±1.2	3.52±0.39	1.79±0.17
Cytochalasin D	12	12	25.9±1.2	3.51±0.19	1.62±0.08*

Rat embryos at the head-fold stage were cultured for 48 hr in rat serum with (Cytochalasin D) or without (Control) cytochalasin D (2×10^{-8} M). Mean±S.D. Student's *t*-test was used for statistical analysis. *, Significantly different from the control value ($P<0.01$).

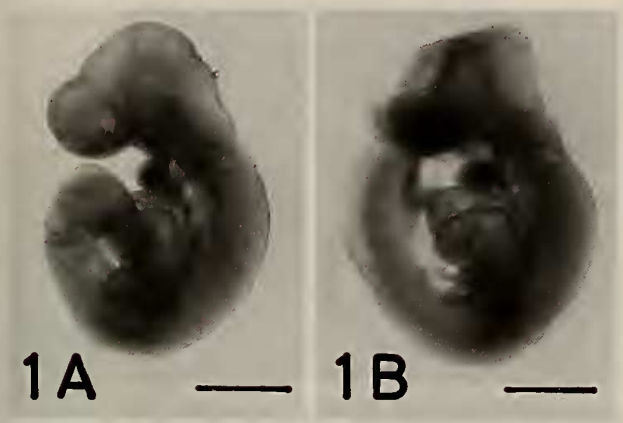

FIG. 1. Gross view of rat embryos at the head-fold stage were cultured in rat serum with (1B) or without (1A) cytochalasin D (2×10^{-8} M) for 48 hr. Fig. 1B shows open an neural tube. Bar, 1 mm.

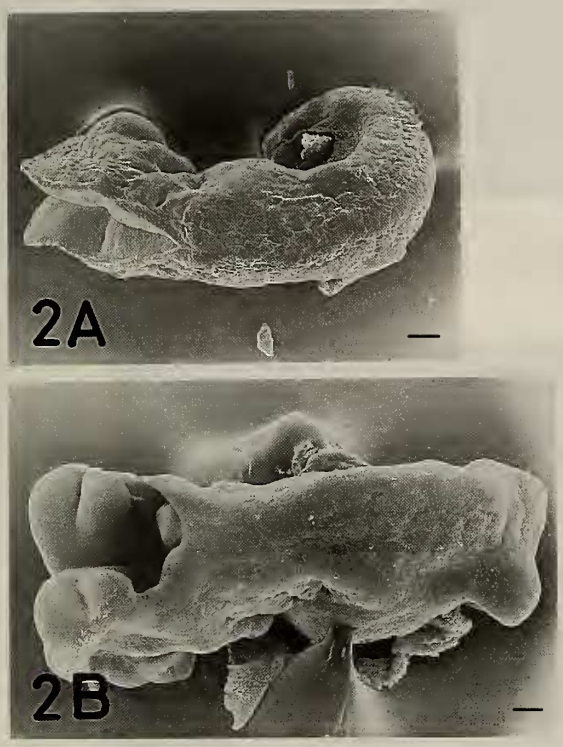

FIG. 2. Scanning electron micrographs of rat embryos. Embryos were cultured for 33 hr (2A) and for 48 hr (2B) in rat serum that contained cytochalasin D (2×10^{-8} M). Fig. 2A shows elevation of the neural folds and Fig. 2B shows eversion of the neural plate. Bar, 100 μm.

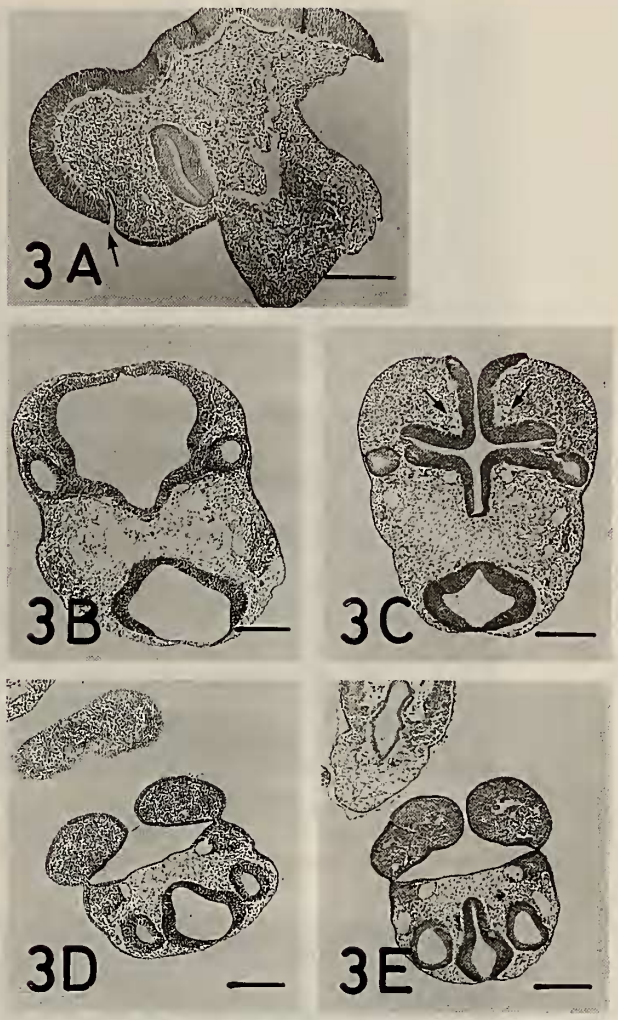

FIG. 3. Anomalies in cytochalasin D-treated rat embryos. Sagittal (3A) and transverse (3B, 3C, 3D and 3E) sections stained with hemalaum. Rat tembryos were cultured in rat serum with (3A, 3C and 3E) or without (3B and 3D) cytochalasin D (2×10^{-8} M) for 48 hr. The diencephalon protrudes between and over the telencephalic vesicles so that parts that normally form the roof of the ventricle are continuous with the skin of the temporal area (3A). An arrow shows the joint that connects the neural plate with the skin. Eversion of diencephalic neuroepithelium is seen in fig. 3C. Arrows indicate the wide space between the neuroepithelium and surrounding mesenchymal cells. The rhombencephalic neuroepithelium continues to the neural groove, the sides of which are closely apposed in the treated embryos (3E). Bars, 250 μm.

in the 24-hr-cultured treated embryos, in which neural plates had opened outwards (Fig. 4B). In 33-hr-cultured control embryos, neural folds fused and the relatively dense staining was observed at the luminal surface of the roof plate. The staining at the luminal surface covered a wide area compared with that at the basal region (Fig. 5A). In contrast, the staining was confined to the edges of the neural folds in the 33-hr-cultured treated embryo (Fig. 5B) in which the neural folds were apposed but not fused. Staining was observed in the roof plate of 36-hr-cultured control embryos (Fig. 6A) but was less in that of 48-hr-cultured control embryos (Fig. 7A) compared with the staining of 36-hr-cultured control embryos. In the treated embryos, neural plates opened outwardly and then everted as time passed. During these processes, staining with rhodamine-conjugated phalloidin was observed at the surface of the neural plates but not at the basal region (Figs. 6B and 7B).

Some parts of the rhombencephalon of the treated

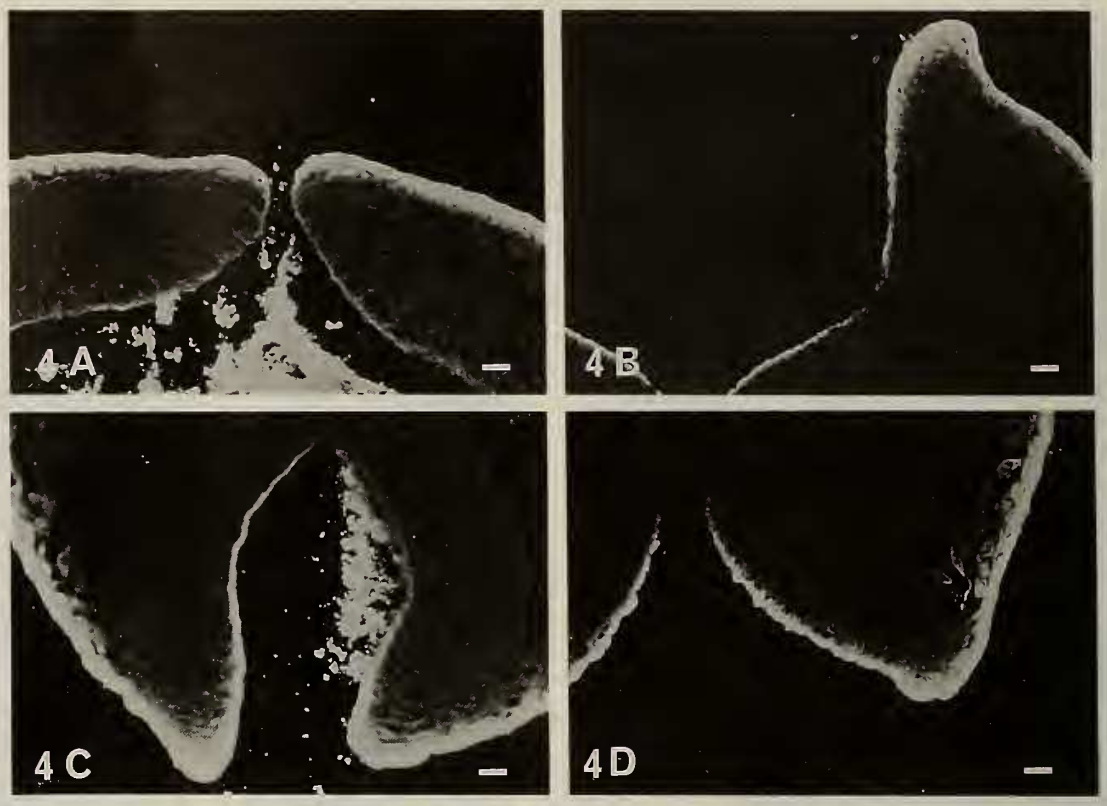

FIG. 4. Distribution of microfilaments in the prosencephalic (4A and 4B) and the rhombencephalic (4C and 4D) neuroepithelium of rat embryos. Transverse sections were stained with rhodamineconjugated phalloidin. Rat embryos at the head-fold stage were cultured for 24 hr in rat serum with (4B and 4D) or without (4A and 4C) cytochalasin D (2×10^{-8} M). Bars, 10 μm.

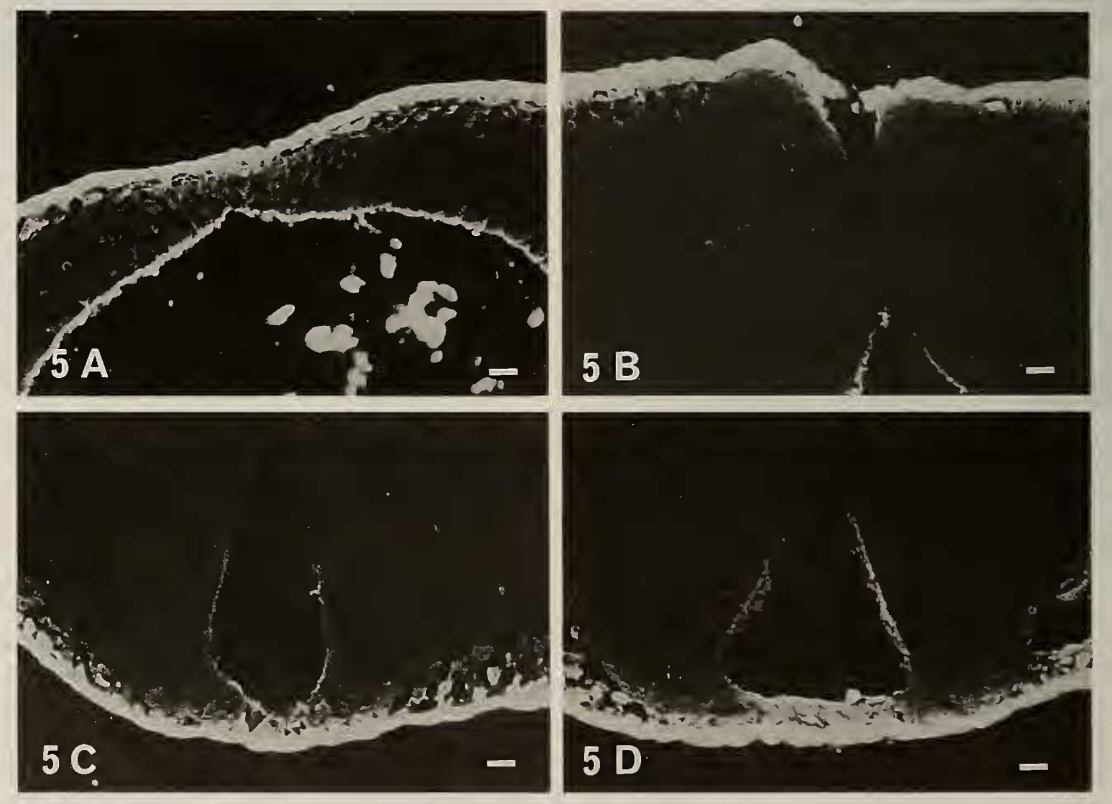

FIG. 5. Distribution of microfilaments in the prosencephalic (5A and 5B) and the rhombencephalic (5C and 5D) neuroepithelium of rat embryos. Transverse sections were stained with rhodamineconjugated phalloidin. Rat embryos as the head-fold stage were cultured for 33 hr in rat serum with (5B and 5D) or without (5A and 5C) cytochalasin D (2×10^{-8} M). Bars, 10 μm.

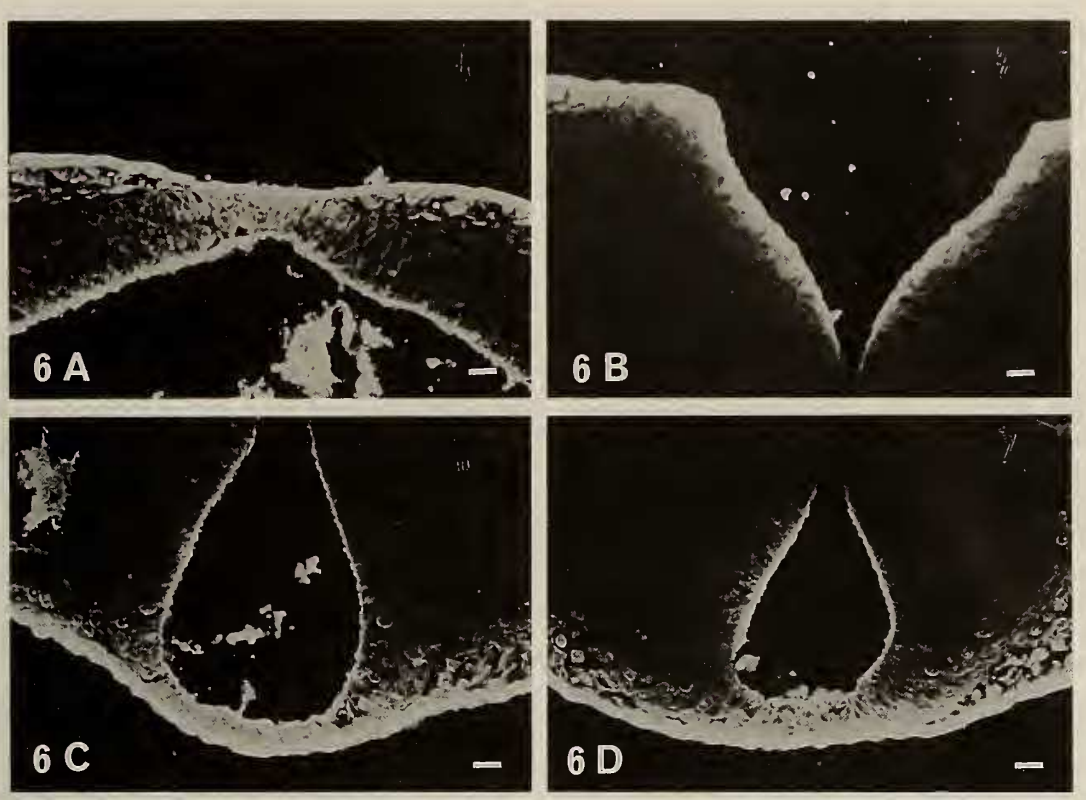

FIG. 6. Distribution of microfilaments in the prosencephalic (6A and 6B) and the rhombencephalic (6C and 6D) neuroepithelium of rat embryos. Transverse sections were stained with rhodamineconjugated phalloidin. Rat embryos at the head-fold stage were cultured for 36 hr in rat serum with (6B and 6D) or without (6A and 6C) cytochalasin D (2×10^{-8} M). Bars, 10 μm.

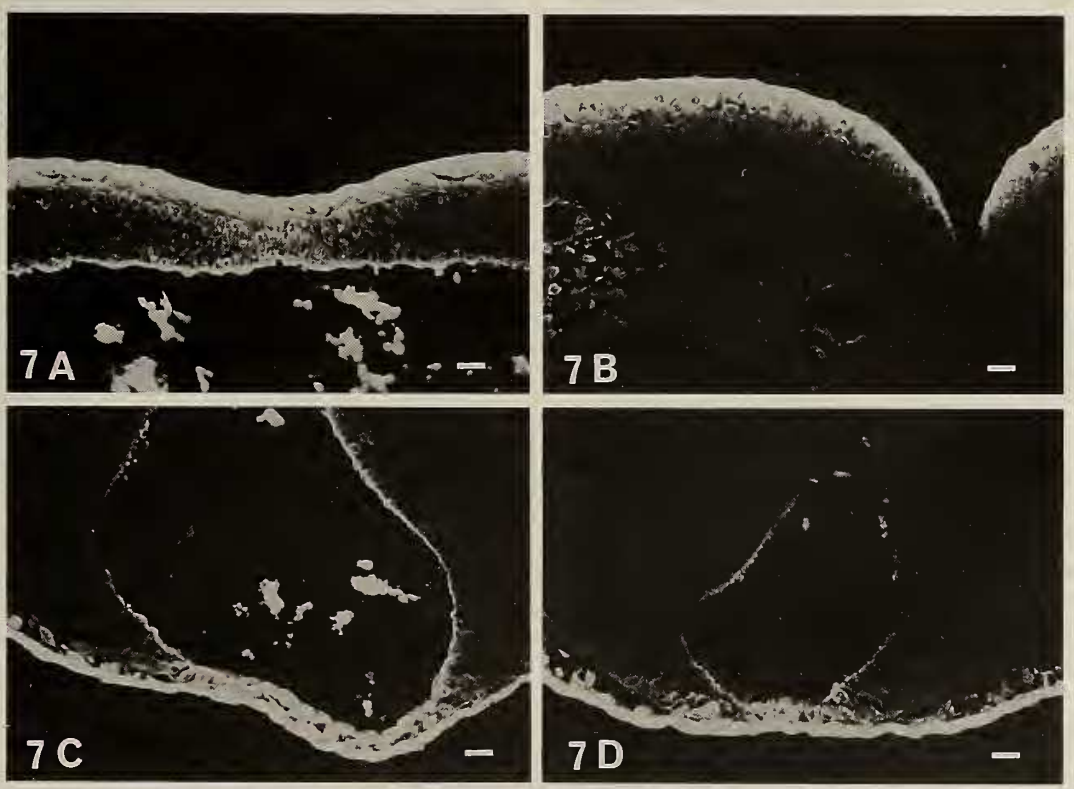

FIG. 7. Distribution of microfilaments in the prosencephalic (7A and 7B) and the rhombencephalic (7C and 7D) neuroepithelium of rat embryos. Transverse sections were stained with rhodamine conjugated phalloidin. Rat embryos at the head-fold stage were cultured for 48 hr in rat serum with (7B and 7D) or without (7A and 7C) cytochalasin D (2×10^{-8} M). Bars, 10 μm.

embryos fused and the staining was detected at the fusing neural folds and in the roof plates as like as control embryos (Figs. 4C, 4D, 5C, 5D, 6C, 6D, 7C and 7D). These figures showed that the area of the staining at the luminal surface of the roof plates spread as the fourth ventricle expanded. As the embryos developed, degree of expansion of the fourth venrticle became different between control and treated embryos and the ventricle was more spacious in the control embryos than in the treated embryos.

DISCUSSION

The results of the present experiments indicate that fusion of the cephalic neural folds in rat embryos can be inhibited by continuous exposure to a relatively low concentration of cytochalasin D (2×10^{-8} M) in vitro. The appearance of the treated embryos resembled to that of the embryos presented by Shepard and Greenaway [22] and by Austin et al. [1] but not those presented by Morriss-Kay and Tuckett [12]. In the investigation of Morriss-Kay and Tuckett [12], collapse of the neural plate to varying extents was apparent in embryos that had been exposed for a short time to a relatively high concentration of cytochalasin D (3×10^{-7} M). This concentration induced death of embryos of C57BL/6 mice within 24 hr although embryos remained alive in 2×10^{-8} M cytochalasin D [17]. It is unclear whether embryos with collapsed neural plates develop exencephaly or not. However, the embryos shown by Shepard and Greenaway [22] and by Austin et al. [1] did develop exencephaly. Therefore, it appears that the embryos obtained in our experimental system should provide a more convenient model for investigations of the mechanism of neurulation.

Neurulation involves formation of the neural plate from the ectoderm, elevation of the neural folds, their apposition and fusion [5, 6, 21]. The results of our experiments indicate that elevation and apposition of the neural folds occur in cytochalasin D-treated embryos but fusion does not. Schoenwolf et al. [20] reported that cytochalasin D did not prevent median furrowing of the neural plate or elevation of the neural folds but did prevent the fusion of neural folds in the chick embryo. Although cytochalasin D produces exencephaly when injected into pregnant mice, the morphological features of such embryos have not been described in detail [1, 22]. It is unclear from the earlier reports whether cytochalasin-induced exencephaly occurs *via* failure of elevation of failure of fusion of the neural folds. However, the fusion process is inhibited both in cadmium-induced exencephaly [28] and in arsenic-induced exencephaly [12]. From these results and the present result, it seems possible that exencephaly occurs as the result of failure of the fusion and not of failure of the elevation or apposition of the neural folds.

Cell shape changes are the basis for morphogenetic movement and the intact microfilaments are required for such changes. The correlation is that in the presence of cytochalasins, microfilaments are disrupted and morphogenesis ceases, and that cytochalasins removal results in microfilament reappearance and resumption of morphogenesis [24]. Cytochalasins reversibly inhibit the growth of microfilaments and degrade preformed microfilaments [2, 24, 29]. Among the cytochalasins, cytochalasin D has a particularly high affinity for contractility-related binding sites but has no effect on hexose transport [3, 11, 26–27]. Thus, the results of our experiments suggest that microfilaments do not play an essential role in the elevation of the neural folds but do play an important role in fusion. Microfilaments are intimately involved in neurulation in amphibian, avian and mammalian embryos [5, 6, 9, 16, 19, 20, 23]. The cited investigations can be divided into three groups in terms of the apparent involvement of microfilaments: microfilaments influenced the elevation of the neural folds [6, 8, 13–14]; they influenced both the elevation and the fusion of the neural folds [9]; and they influenced the fusion of the neural folds [20]. Our study falls into the third group. Although interpretation of the various earlier results is complex, it is possible that a relatively high concentration of cytochalasin D (3×10^{-7} M) affects the elevation and apposition of the neural folds while a relatively low concentration of the drug (2×10^{-8} M) does not affect the elevation but affects the fusion of the neural folds.

The results of the present experiments indicated that when the fusion of the neural plates failed the staining with rhodamine-conjugated phalloidin appeared at the surface of the end region of the neural plates, while the staining was observed at the basal region of the end region of the neural plates in the process of the successful fusion. These results suggest that the state of microfilaments at the end region of the fusing neural plates is intimately related to the fusion. Delicate changes in the distribution of microfilaments may result in changes in cell shape that cause the fusion of the neural plates. The results of our experiments also show that the distribution of microfilaments changed with the moulding of the cephalic neural tube, suggesting that microfilaments play a role not only in fusion but also in the moulding of the cephalic neural tube.

Eversion of the telencephalic neural plates followed failure of fusion of neural folds. Our experimental results revealed that microfilaments at the luminal surface was not observed at the time of apposition in the treated embryos then it appeared with eversion of the neural plates. In the present time, why appearance of microfilaments was not inhibited in the treated embryos is unclear. The various cell types respond with distinctly differing sensitivities to cytochalasins [4] and cells at the luminal surface may be relatively more resistant than the fusing cells, and/or that cytochalasin D may be eliminated from cells at the luminal surface by an active pump, such as the transmembrane pump in tumor cells [18].

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